

MINIREVIEW

Recent Advances in Determining the Pathogenesis of Canine Monocytic Ehrlichiosis

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Canine monocytic ehrlichiosis (CME) is a potentially fatal tick-borne disease caused by the rickettsia *Ehrlichia canis* (16). The etiologic agent was first recognized in Algeria in 1935 (8). Since then, it has been reported worldwide, causing extensive morbidity and mortality among domestic dogs and other canids (11, 28, 51). The principal vector of CME is *Rhipicephalus sanguineus* (11). Recently, it has been shown experimentally that *Dermacentor variabilis* is also capable of transmitting *E. canis* (24).

The pathogenesis of CME consists of an incubation period of 8 to 20 days, followed sequentially by acute, subclinical, and in some cases chronic phases. The disease may be manifested by a wide variety of clinical signs of which depression, lethargy, weight loss, anorexia, pyrexia, lymphadenomegaly, splenomegaly, and bleeding tendencies are the most common. Principal hematologic abnormalities include thrombocytopenia, mild anemia and mild leukopenia during the acute stage, mild thrombocytopenia in the subclinical stage, and pancytopenia in the severe chronic stage. The main biochemical abnormalities include hypoalbuminemia, hyperglobulinemia, and hypergammaglobulinemia (16).

CME has been researched extensively in the last decade, and special efforts have been made to elucidate the pathogenesis of the disease. Better understanding of major mechanisms involved in the pathogenesis of the disease may assist clinicians in understanding the disease process and providing appropriate treatment, affording a better prognosis to their patients. In the light of the recent emergence of similar ehrlichial pathogens that infect human patients, the understanding of pathogenic processes in CME may contribute to the understanding of human monocytic ehrlichiosis and human granulocytic ehrlichiosis. This article reviews recent investigations in the pathogenesis of CME with special reference to platelet disorders and serum protein alterations, the principal hematological and biochemical abnormalities in CME, respectively. Host immune response in both acute and persistent *E. canis* infection is discussed and is proposed to be involved in the pathogenesis of disease manifestations.

PLATELET DISORDERS

Thrombocytopenia is considered to be the most common and consistent hematological abnormality of dogs naturally or

experimentally infected with *E. canis* (56). The thrombocytopenia in CME is attributed to different mechanisms in the different stages of the disease. Mechanisms thought to be involved in the pathogenesis of thrombocytopenia in the acute phase of the disease include increased platelet consumption due to inflammatory changes in blood vessel endothelium, increased splenic sequestration of platelets, and immunologic destruction or injury resulting in a significantly decreased platelet life span (27, 43, 54). Studies using radioisotopes have shown that platelet survival time decreased from a mean of 9 days to 4 days, 2 to 4 days after infection with *E. canis* (54). In addition, a platelet migration inhibition factor was isolated and characterized. This factor is proposed to play a role in enhancing platelet sequestration and stasis, leading to reduced peripheral-blood platelet counts (1).

Demonstration of serum platelet-bindable antiplatelet antibodies (APA) in dogs after experimental infection with *E. canis* supports the assumption that immune destruction may also contribute to the pathogenesis of thrombocytopenia in acute ehrlichiosis (14, 56). The earliest detection of APA was made on day 7 postinfection (p.i.) in one of six dogs, on day 13 in three, and on day 17 in the two remaining dogs (14). APA have also been demonstrated in 80% of serum samples of human patients infected with granulocytic ehrlichiosis (60). The stimulus for the production of these autoantibodies is not fully understood; however, two theories have been proposed. The early appearance of APA prior to appearance of *E. canis* antibodies suggested that B cells carrying natural autoantibody receptors may be induced to undergo proliferation and maturation by interaction with ehrlichial antigens which are antigenically similar to self antigens. The alternative theory proposed that APA develop secondarily to platelet components undergoing destruction and massive release of platelet structural proteins brought about by nonimmunologic platelet destruction (56). Complement consumption was shown to occur during the thrombocytopenic phase of acute ehrlichiosis, and partial decompensation of infected dogs' sera moderated the severity of the thrombocytopenia, further substantiating the argument for an immunopathologic component in the pathogenesis of thrombocytopenia in CME (33). Concurrently with the development of the thrombocytopenia during the acute phase, a significant increase in the mean platelet volume is usually seen and reflects active thrombopoiesis (56). In the severe chronic phase of disease, decreased platelet production due to bone marrow hypoplasia is considered to be the reason for the thrombocytopenia (61). In this stage, dogs frequently exhibit pancytopenia as a result of this hypoplastic bone marrow, further complicating their clinical status.

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Platelet adhesiveness was shown to decrease in dogs acutely infected with *E. canis* (33). Furthermore, sera of *E. canis*-infected dogs were shown to inhibit platelet aggregation when incubated with platelets of a healthy dog, seronegative for CME. These findings suggest that platelet dysfunction may occur in the acute stage of CME and, together with thrombocytopenia, may be a factor contributing to the bleeding tendency observed in the disease (13). The presence of maximal concentrations of serum APA concurrent with platelet dysfunction (in days 17 to 24 after experimental infection) suggested that APA played a role in causing platelet dysfunction in the acute stage of canine ehrlichiosis. Interaction of APA with platelet membrane glycoproteins was proposed to cause the platelet dysfunction (13).

SERUM PROTEIN ALTERATIONS

Hypoalbuminemia, hyperglobulinemia, and hypergamma-globulinemia are the predominant biochemical abnormalities found in dogs infected with *E. canis* (5, 12, 58). The hypoalbuminemia seen in CME may be the consequence of peripheral loss of albumin to edematous inflammatory fluids as a result of increased vascular permeability (61), blood loss, or decreased protein production due to concurrent mild liver disease (45), or it may be due to minimal-change glomerulopathy (6). As albumin synthesis is regulated by oncotic pressure (53), the decrease in albumin concentrations may act as a compensatory mechanism for the hyperglobulinemic state, thereby maintaining the oncotic pressure and preventing an increase in blood viscosity (61).

The hypergammaglobulinemia in CME is usually polyclonal. Monoclonal gammopathy rarely occurs and may result in hyperviscosity and associated clinical manifestations (12, 22, 42). Gamma globulin concentrations increase during the febrile phase of canine ehrlichiosis and persist during the subclinical and chronic phases of the disease (51). There is a poor correlation between the gamma globulin concentrations and specific *E. canis* antibody titers (12, 45, 58). The poor correlation between these two parameters and the polyclonal gammopathy recorded to occur in most sick dogs suggest that nonspecific antibody production is induced by *E. canis* and that the anti-*E. canis* antibodies are not the main source of gamma globulins contributing to the hypergammaglobulinemia. This phenomenon is known to occur in other diseases with prolonged antigenic stimulation (55) and suggests an exaggerated immune response to *E. canis* with inadequate effectiveness (45).

α_2 - and β_2 -globulin concentrations were also found to increase in infected dogs (12). In order to elucidate whether acute-phase protein responses occur in dogs infected with *E. canis*, C-reactive protein, a β -globulin, has been studied (50). Levels of C-reactive protein were found to rise gradually between days 4 and 6 p.i. and declined to preinfection levels by day 34, substantiating the hypothesis that the acute-phase protein response occurs in the acute phase of CME. The increase in α_2 -globulin concentrations may be the consequence of tissue damage and inflammation, as it has previously been demonstrated that synthesis of α_2 -globulin by the liver was stimulated by leukocyte endogenous mediators in response to tissue damage and inflammation (29).

IMMUNE RESPONSE

Increasing evidence supports the assumption that immune mechanisms are involved in the pathogenesis of acute CME. This evidence includes extensive plasma cell infiltration of parenchymal organs, the occurrence of polyclonal hypergamma-

globulinemia that does not correlate with specific *E. canis* antibody titers, positive Coomb's and autoagglutination tests, and the induction of APA production following experimental *E. canis* infection in dogs (12, 21, 61).

There is no predilection for age or sex, and all breeds may be infected with CME (15); however, German shepherd dogs (GSD) seem to be more susceptible to CME than other breeds (15, 38, 51). Moreover, the disease in GSD is more severe and has a poorer prognosis than in other breeds (15). Differences in breed susceptibility can be attributed to breed differences in the ability to mount adequate cellular and/or humoral immune responses. It has been documented that the cellular immune response against *E. canis* is depressed in GSD compared with beagle dogs (38). In the same study, no significant differences in the humoral response were noted between the two breeds. These findings suggest that the cellular immune response is the more important component of the immune system providing protection against *E. canis*. In experimentally infected dogs, persistent high antibody titers following treatment and elimination of the rickettsia were shown to be of no protective value when dogs were challenged with homologous or heterologous *E. canis* strains (4, 51). Thus, the humoral immune response does not appear to play an important role in protection against *E. canis*; conversely, it has been proposed to contribute to the pathogenesis of the disease (21, 51).

A state of premunity (protective immunity) is thought to occur in dogs subclinically infected with *E. canis* and also in infected dogs after short-term treatment with oxytetracycline (3, 9, 30, 51). It seems that protective immunity in CME is maintained primarily via the cellular immune response rather than the humoral response.

The humoral response to *E. canis* may be studied by serum protein electrophoresis and serological testing using the immunofluorescence antibody test, enzyme-linked immunosorbent assay, and Western immunoblot. Immunoblot analysis showed that immune sera obtained from *E. canis*-infected dogs react with a wide variety of *E. canis* proteins in the range of 21 to 160 kDa (20, 23, 39, 48, 50). The strongest immune reaction has been shown to a protein of approximately 27 to 30 kDa (4, 20, 36, 49). Results of a comparative international survey indicated that antigenic heterogeneity may exist among *E. canis* organisms in different regions of the world (20, 47). A similar heterogeneity was reported in the antibody response to *Cowdria ruminantium* (of the *Ehrlichieae* tribe). An immunodominant conserved antigen of approximately 32 kDa (Cr32) has been found in *C. ruminantium* (26), and this antigen was later renamed MAP1 (2), after it became clear that its molecular size varied not only according to the geographical origin of the strain but also according to the electrophoretic conditions. This antigenic diversity may be one of the reasons for the variety in the clinical manifestations of CME in different geographical regions. This hypothesis is substantiated by the fact that heterologous challenge of dogs with the North Carolina isolate of *E. canis* 90 days following challenge with the Florida strain (after treatment and elimination of the rickettsia) resulted in increased disease severity in comparison with that induced by homologous challenge (4).

Host response to *E. canis* infection was suspected to play an important role in the pathogenesis of the disease, and alteration of the host's immune system by using cyclophosphamide and antilymphocyte serum has proven to alter the pathologic and clinical manifestations of experimental *E. canis* infection (46). To determine the role of the spleen in the pathogenesis of CME, the effect of splenectomy on the course of the acute phase of experimental CME was investigated (19). The clinical and hematological findings of the study indicated that the

disease process was considerably milder in the splenectomized dogs than in the intact dogs. There did not appear to be any difference in the time of appearance or in the titer of anti-*E. canis* immunoglobulin G antibodies between splenectomized and intact dogs throughout the course of the study. During the acute stage, food consumption was significantly higher in the splenectomized group than in the intact group. During this period, significantly higher body temperatures were measured in the intact group compared to the splenectomized group. The hematocrit, erythrocyte counts, hemoglobin concentrations, and platelet counts were significantly higher in the splenectomized group than in the intact group during the whole course of the study. The spleen plays a major role in the pathogenesis of immune-mediated diseases, and in cases refractory to medical treatment splenectomy may be indicated (31). Removal of the dominant organ producing antibodies and elimination of one of the major sites of the monocytic phagocytic system are considered the main objectives achieved by splenectomy. The spleen is a major site for the synthesis of tuftsin and properdin, which serve as opsonins and promote phagocytosis. The spleen is also an important site for the synthesis of complement components. By elimination of the splenic macrophages and reduction of complement components and opsonins, postsplenectomy phagocytosis is compromised (10, 32). The results of our recent study suggest that the spleen plays a key role in the pathogenesis of CME and further support the notion that immune mechanisms are involved in the pathogenesis of CME (19).

PERSISTENCE OF INFECTION

Following the acute phase of the disease, *E. canis* infection may persist after spontaneous clinical recovery or ineffective treatment, and such animals may enter the subclinical stage of CME (17). Mild hematological abnormalities have been reported to occur in the subclinical phase of disease in experimentally and naturally infected dogs. These abnormalities include mild thrombocytopenia and a significant decrease in leukocyte counts compared to preinfection values, due to a reduction in the neutrophil counts. However, the dogs were neither leukopenic nor neutropenic during this stage (7, 57). These findings suggest that the mild thrombocytopenia and reduced leukocyte counts may be indicative of continued pathological changes and therefore should not be overlooked, as these animals may be subclinical carriers of *E. canis*.

In a 3-year follow-up study, ehrlichial DNA was amplified by PCR from four of six clinically healthy untreated dogs 34 months after experimental infection with *E. canis*. At this stage, the two PCR-negative dogs had platelet counts within the reference range, while three of the four PCR-positive dogs were thrombocytopenic. Furthermore, one of the PCR-negative dogs was seronegative and the other had the lowest *E. canis* antibody titers. These findings proved that clinically healthy dogs in the subclinical phase of CME are carriers of the rickettsia, that infection with *E. canis* may persist for years without development of the chronic clinical disease, and that some dogs can eliminate the parasite and recover from CME without medical treatment (as occurred in two of the six dogs) (17, 18). Asymptomatic persistent infection (for 1 year) of a woman with a rickettsia named Venezuelan human ehrlichia (VHE) was also reported. The VHE was found to be closely related to the Oklahoma and Florida strains of *E. canis*, with 99.9% similarity in the base sequence of the 16S rRNA genes. The VHE was proposed to be a new strain or a subspecies of *E. canis* (41).

As premunition requires a carrier state, the finding of our

subclinical study substantiates the possibility of existence of premunition in subclinical CME (17). We extracted DNA from blood, bone marrow, and splenic aspirates from each of six dogs. Ehrlichial DNA was retrieved from the spleens of all four PCR-positive dogs but from bone marrow and blood samples of only two. These findings indicate the importance of the spleen in the pathogenesis and establishment of the disease. They also correlate with the fact that splenectomized dogs experimentally infected with *E. canis* suffered more mildly from the acute disease, probably due to removal of a major organ in which colonization by the parasite takes place (19). These findings also suggest that of the spleen, bone marrow, and blood, the spleen is probably the last to harbor *E. canis* parasites during recovery. It was suggested that splenic aspirates are the best source of DNA for PCR used in diagnosing an *E. canis* carrier state during subclinical ehrlichiosis. It was also suggested that PCR performed with DNA extracted from blood or bone marrow samples would not give correct results and may even be misleading. In addition to PCR, Western immunoblot analysis may assist in determination of the stage of infection. It has been shown that during the acute phase (days 7 to 30 p.i.), untreated dogs produce antibodies against low-molecular-mass major proteins (≤ 30 kDa). However, antibodies to higher-molecular-mass proteins (>30 kDa) are more easily detected in persistent infections (39, 48). Tissue culture and/or PCR may give the most accurate results in determining the persistence of ehrlichial infection (4, 18, 23). In our experience, the indirect immunofluorescence antibody test is not a reliable method to determine persistence of infection or success of treatment during or shortly after treatment, as titers have been shown to remain high for long periods after elimination of the parasite (18). Microscopic evaluation of Giemsa-stained smears prepared from blood, bone marrow, and splenic aspirates was shown to be an insensitive technique for the diagnosis of subclinical CME. It is probable that the number of parasites in a subclinically infected animal is too small to be observed on microscopic examination of blood, bone marrow, or splenic smears (18).

Some dogs suffering from the subclinical stage of CME can develop the severe life-threatening chronic stage of the disease. The conditions that lead to the development of the chronic stage are not fully understood; however, they may be related to the breed, the immune status of the animal, stress conditions, coinfections with other parasites, geographical location, the strain of the parasite, or persistent reinfection (4, 16, 20). The risk of developing the chronic, severe form of the disease should be considered in subclinical cases and should not be ignored. Diagnosing and treating these subclinical dogs is recommended in order to prevent further progression of the disease (18).

FUTURE DIRECTIONS

The pathogenesis of the acute phase of CME has been investigated extensively, and recent research has added to our knowledge of the subclinical phase. However, little is known regarding the pathogenesis of the chronic phase of CME. This phase of the disease has not yet undergone comprehensive investigation as no suitable model for the chronic disease has been developed to date, nor has it been possible to consistently induce the chronic disease in experimentally infected dogs. Therefore, it is proposed that clinical trials using dogs with the naturally occurring chronic disease should be undertaken. Better understanding of the conditions that lead to the development of this stage and understanding of the pathogenesis of the bone marrow depression in this stage may aid in develop-

ment of better treatment protocols and result in an improved prognosis.

Investigation of the cellular immune response to *E. canis*, the cytokines involved, and their role in the pathogenesis of CME warrants further investigation into the different phases of the disease. Understanding the immune mechanisms and determining the factors involved in the pathogenesis of each phase are essential. These findings may also resolve the debate on the use of immunosuppressive drugs in the different phases of CME and may also promote research on vaccine production.

No successful vaccine for CME or for any human or canine ehrlichial disease has yet been developed. In a series of immunization studies using inactivated cell culture-derived *E. canis* antigen, fortified by adjuvants, good levels of antibody response were induced. However, when dogs were challenged, the clinical manifestation of the disease in the immunized animals appeared more fulminating than in the nonimmunized control dogs (51). Conversely, in a recent study, five German shepherd dogs were immunized with inactivated *E. canis* in combination with the adjuvant Quil A, while two control dogs were injected only with the adjuvant. In vitro proliferation assays using peripheral blood mononuclear cells, high indirect immunofluorescent-antibody titers, and Western blotting demonstrated induction of the cellular and humoral immune responses following immunization. Challenge infection with live *E. canis* resulted in milder clinical and hematological signs in the immunized dogs than in the control dogs. The authors suggested that partial protection was achieved by the immunization with the inactivated *E. canis* organisms (34). Attenuated and inactivated vaccines derived from the closely related ehrlichia *C. ruminantium* have been shown to produce protection in small ruminants (25, 35). Furthermore, a MAP1-based DNA vaccine prepared from *C. ruminantium* was shown to be efficient in protecting up to 88% of mice on challenge with a lethal dose of the homologous strain (37). Recently, the 28- and 30-kDa surface-antigen genes of *E. canis* were cloned and sequenced (40, 47). This might eventually result in the development of a recombinant vaccine against CME. However, this may not be easy, as antigenic variation between strains from different geographical regions may exist (20). The significance of such a finding with regard to vaccine production has to be further investigated as it may complicate the development of recombinant vaccines based on the major outer membrane proteins (20, 47). Development of an *E. canis* vaccine, which may be used in the prophylactic program to prevent *E. canis* infection in dogs and other wild canids, will have significant socioeconomic implications as well as animal welfare benefits. Successful development of a vaccine will serve as a model for the development of other antiehrlichial vaccines, especially against the life-threatening human ehrlichial diseases.

To date, tick control remains the most effective preventive measure against *E. canis* infection. The most acceptable method is the conventional use of acaricides. An alternative novel method for tick control used with large animals is the antitick vaccine. The protective antigen Bm86 was identified from the guts of semiengorged adult female *Boophilus microplus* ticks and was obtained by recombinant-DNA technology (44, 59). Vaccines containing this antigen were released to the market and were shown to be effective in field trials (52). The concept of antitick vaccination of pet animals has not been investigated. With respect to CME, development of a vaccine against *R. sanguineus* warrants future investigation.

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